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# Inhibition and interaction of cytochrome P450 of *Candida krusei* with azole antifungal drugs

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*Candida krusei* has become an increasingly important invasive pathogen, particularly in AIDS patients and is highly resistant to fluconazole. *In vitro* growth inhibition studies revealed that fluconazole and ketoconazole were approximately 800- and 19-fold less inhibitory than itraconazole. The inhibition and interaction of itraconazole, fluconazole and ketoconazole with the sterol 14 $\alpha$ -demethylase of *C. krusei* was studied using *in vitro* ergosterol biosynthesis and difference spectroscopy, respectively. Both itraconazole and ketoconazole inhibited *in vitro* ergosterol biosynthesis at lower concentrations than fluconazole. All three drugs interacted with microsomal P450 and interfered in the binding of carbon monoxide to P450 in direct proportion to their inhibitory effect on ergosterol biosynthesis in cell-free extracts. The slightly lower affinity of sterol 14 $\alpha$ -demethylase for fluconazole compared with itraconazole and ketoconazole is only partially responsible for poor activity of fluconazole on *C. krusei*.

**Keywords** azole antifungals, cytochrome P450, *C. krusei*

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## Introduction

The azole antifungal agents have been used with considerable success in both agriculture and medicine. They act primarily through inhibiting the sterol 14 $\alpha$ -demethylation reaction, a key step in ergosterol biosynthesis, by interacting with the cytochrome P450 (sterol 14 $\alpha$ -demethylase [P450<sub>14dm</sub>]) which catalyzes the reaction. The consequence of this inhibition is a depletion of ergosterol and corresponding increase in 14 $\alpha$ -methyl sterols, which is thought to disrupt membrane structure and function, leading to an inhibition of growth [1].

Serious infections caused by fungi, particularly *Candida* spp., have increased steadily in the past two decades due to increased frequencies of organ transplantation, chemotherapy for cancer treatment and HIV infection [2-4]. Fluconazole, a triazole antifungal, has proved to be a valuable drug in treatment of many *Candida* infections [5]. However, *Candida krusei* is inherently resistant to fluconazole [6-8]. In contrast to fluconazole, itraconazole

and ketoconazole are active against *C. krusei* [8]. Recent studies showed that resistance of *C. krusei* to fluconazole was correlated both with low intracellular accumulation of this drug and also with low affinity for the sterol 14 $\alpha$ -demethylase [9].

Previous study on the affinity of azole antifungals indicated that they were more tightly bound to the fungal P450 than to plant and animal P450s [10]. Studies with *C. albicans* and yeast cytochrome P450 showed that relative affinity is likely to be determined by the interaction of N-1 substituent group and the apoprotein of the P450 involved [11]. Similar interaction studies were also carried out with microsomal P450s from the filamentous fungi like *Aspergillus fumigatus* and *Penicillium italicum* [12,13]. However, isolation of cytochrome P450 and interactions studies with azole drugs have not been carried out so far in *C. krusei*.

This paper describes a method for isolating microsomal P450 and the effect of three systemic azole antifungal drugs on cell growth, cell-free sterol biosynthesis and comparison of inhibitory activity with the affinity for microsomal P450.

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## Materials and methods

### Strains

*Candida krusei* ATCC 6258 strain was used.

### Chemicals

Unless specified all chemicals were obtained from Sigma Chemical Company (Poole, UK). Fluconazole was purchased from Pfizer (Sandwich, UK) and itraconazole and ketoconazole from Janssen Pharmaceutics (Beerse, Belgium). [2-<sup>14</sup>C] mevalonate, dibenzethylenediamine salt (specific activity 53 mCi mmol<sup>-1</sup>) was obtained from Amersham International (Bucks, UK). All the azole drugs used in this study were dissolved in dimethyl sulphoxide (DMSO) to obtain a stock solution of 10 mM for fluconazole and 1 mM for itraconazole and ketoconazole.

### Growth inhibition studies

*Candida krusei* cells were grown at 37 °C on Sabouraud glucose medium (Difco, Basingstoke, UK) containing agar (2%, w/v, Difco) in Petri dish plates and maintained by storing at 4 °C. The growth inhibition studies were carried out using RPMI-1640 medium (Sigma) buffered with 0.165 M MOPS, pH 7.0. The inoculum was prepared by suspending the cells obtained from the plates incubated at 37 °C for 48 h in RPMI-1640 medium at  $2 \times 10^4$  cells per ml. Stock solutions of azole drugs were diluted with RPMI-1640 medium to yield a 640 µM (fluconazole) or 64 µM (itraconazole and ketoconazole) start solution and these start solutions were double diluted with RPMI-1640 medium to give a range of drug concentrations from 640 to 1.2 µM (fluconazole) and 64 to 0.12 µM (itraconazole and ketoconazole). One millilitre of inoculum was added to 1 ml RPMI-1640 (containing various concentrations of azole drug) contained in a 60 ml Sterilin container to give a final inoculum of  $10^4$  cells per ml and drug concentrations from 320 to 0.6 µM for fluconazole and 32 to 0.06 µM for itraconazole and ketoconazole. Treatment with azole antifungal compounds was carried out in triplicate for 48 h at 37 °C, 150 rev min<sup>-1</sup> and growth was assessed by cell counts. Cultures grown without azole were used as controls. MIC tests carried out as described by Wardle *et al.*; a modification of the M27-P method of the National Committee for Clinical Laboratory Standards yielded identical results [14]. Each test was repeated at least two times and MICs obtained were identical.

### Cell-free extract preparation

After inoculating 0.5% of overnight culture grown in PYG medium (10 g of polypeptone, 10 g of yeast extract

and 40 g glucose per litre) into fresh PYG medium and growing at 150 rev min<sup>-1</sup>, at 37 °C for 16 h, cells were washed and resuspended in 100 mM potassium phosphate buffer containing 5 mM MgCl<sub>2</sub>, 30 mM nicotinamide and 5 mM glutathione (buffer A). Resuspended cells were mixed with equal volume of 20 g of glass beads (0.45–0.50 mm) and homogenized using a Braun disintegrator (Braun GmbH, Mesungen, Germany) operating at 4000 rev min<sup>-1</sup> with 4 × 30 s bursts with liquid carbon dioxide cooling. Cell-free extracts were obtained following centrifugation at 1500 g [9].

### Sterol biosynthesis in cell-free extract

Fluconazole, ketoconazole and itraconazole inhibition of P450<sub>14dm</sub> was investigated by assessing the sterol biosynthesis in cell-free extracts of *C. krusei* according to the method described in the previous study [15]. The reaction mixture consisting of cell-free extract (924 µl ml<sup>-1</sup>, 10–15 mg ml<sup>-1</sup> protein concentration), cofactor solution (50 µl; containing 1 µmol NADP, 1 µmol NADPH, 1 µmol NAD, 3 µmol glucose 6-phosphate, 5 µmol ATP and 3 µmol reduced glutathione in distilled water, adjusted to pH 7.0 by adding 10 M KOH), divalent cation solution (10 µl of 0.5 M MgCl<sub>2</sub> and 5 µl of 0.4 M MnCl<sub>2</sub>), solution of azole antifungal compound (1 µl of various concentrations ranging from 1 to 1000 µM), [2-<sup>14</sup>C]mevalonate (10 µl; 0.25 µCi) was incubated for 2 h at 37 °C, 110 rev min<sup>-1</sup> and then the reaction was stopped by adding 1 ml of freshly prepared saponification reagent (15% [w/v] KOH in 90% [v/v] ethanol). Non-saponifiable lipids (sterols and sterol precursors) were extracted with 2 × 3 ml petroleum ether (bp 40–60 °C) and dried under nitrogen. The non-saponifiable lipids were dissolved in 100 µl of petroleum ether, applied to silica gel thin layer chromatography plates (ART 573, Merck) and developed using toluene:diethyl ether 9:1 (v/v). Radioactive sterols were located by autoradiography and excised for scintillation counting. The production of ergosterol was assessed for inhibition as described previously [15]. Experiments were performed in triplicate and IC<sub>50</sub> values for inhibition of ergosterol biosynthesis were calculated.

### Isolation of microsomal cytochrome P450

The cell-free extract obtained by the above procedure was centrifuged at 15 000 g for 20 min to remove mitochondria. The supernatant was then centrifuged at 105 000 g for 90 min to pellet the microsomal fraction. The microsomal pellet was resuspended in buffer A containing 20% (v/v) glycerol using a Potter–Elvehjem glass homogenizer and stored at –80 °C until further use.

### Protein content estimation

Protein content of cell-free extract and microsomes were estimated using the bicinchoninic acid (BCA) protein assay kit (Sigma). To 100  $\mu$ l sample 2 ml of freshly prepared BCA reagent was added, mixed and incubated at 37 °C. After 30 min the absorbance was measured at 562 nm in a spectrophotometer (Philips PU8800) using polystyrene cuvettes. Bovine serum albumin used as the standard.

### Cytochrome P450 content estimation

Cytochrome P450 concentration was measured as described previously [16]. Microsomal suspensions reduced with sodium dithionite were transferred to two quartz cuvettes and the baseline was recorded using Philips PU8800 UV/VIS scanning spectrophotometer. The contents in the sample cuvette were bubbled with carbon monoxide for 45 s at a rate of one bubble per second and the difference spectra recorded.

### Type II binding spectra

Microsomal suspensions (100 nm) were transferred into each of two quartz cuvettes and a baseline was recorded from 390 to 500 nm. Difference spectra were then recorded after incremental additions of the azole antifungal compound to the test cuvette and an equal volume of solvent to the reference. The final concentration of solvent (DMSO) did not exceed 1% (v:v) [12].

### Carbon monoxide displacement spectra

These studies were carried out as described previously [12]: 100 nm microsomal P450 was incubated on ice with 5  $\mu$ M azole (1%, v:v, solvent [DMSO] in control experiment) compound for 2 min and then reduced with sodium dithionite. The contents were transferred to two quartz cuvettes and the cuvettes were then placed in the sample and reference housings of the spectrophotometer. After recording the baseline between 400 and 500 nm, the contents in the sample cuvette were gently bubbled with carbon monoxide for 45 s and difference spectra recorded at intervals between 1 and 60 min.

## Results

### Effects of antifungal agents on growth

The effect of itraconazole, fluconazole and ketoconazole on cell growth was determined and MIC values are shown in Table 1. Fluconazole and ketoconazole were approximately 19- and 800-fold less inhibitory than itraconazole (based on molar comparison).

Table 1 Minimum inhibitory concentration (MIC) of different azole antifungal drugs for *C. krusei* ATCC 6258

Azole drug	MIC	
	$\mu$ M	$\mu$ g ml <sup>-1</sup>
Itraconazole	0.4	0.3
Ketoconazole	7.5	4.0
Fluconazole	320.0	99.8

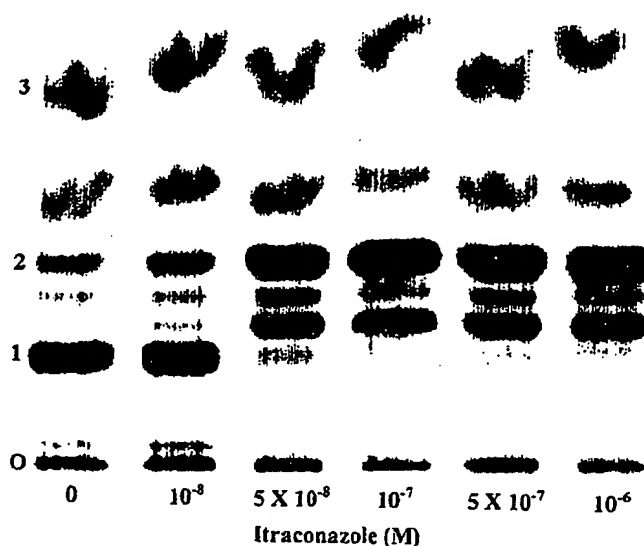


Fig. 1 Autoradiogram of a TLC separation of non-saponifiable lipids synthesized from [2-<sup>14</sup>C]mevalonate in cell-free extracts of *C. krusei*. Band 1, 2 and 3 contain ergosterol, 14 $\alpha$ -methyl sterols and squalene respectively (O, origin).

### Effect of azole antifungal agents on sterol synthesis in cell-free extract of *C. krusei*

Inhibition of sterol 14 $\alpha$ -demethylase activity by azole antifungal drugs was assessed through the study of incorporation of [2-<sup>14</sup>C]mevalonate into ergosterol in cell-free extract of *C. krusei*. When cell-free extract was incubated with [2-<sup>14</sup>C]mevalonate, approximately 20% of total radioactivity in the assay was recovered in the non-saponifiable lipids (NSLs). Sterols were separated from other NSLs components and fractionated into ergosterol and 14 $\alpha$ -methyl sterols by one-dimensional thin layer chromatography (TLC) [15]. Figure 1 shows an autoradiogram of a TLC plate on which NSLs extracted from the cell-free extracts containing different concentrations of itraconazole incubated with [2-<sup>14</sup>C]mevalonate were separated. By comparing the  $R_f$  values of authentic standards, compounds in band 1, 2 and 3 were assigned as ergosterol, 14 $\alpha$ -methyl sterols and squalene, respectively, and remaining bands were either intermediates or

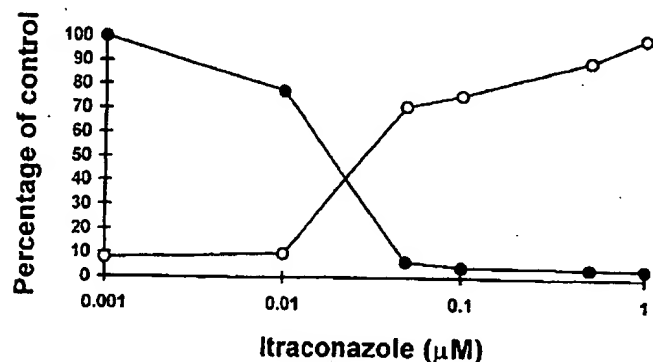


Fig. 2 Effect of itraconazole on incorporation of [2-<sup>14</sup>C]mevalonate into ergosterol (●) and 14α-methyl sterols (○) of cell-free extracts of *C. krusei*.

Table 2 Concentrations of azole drugs required to cause 50% inhibition (IC<sub>50</sub>) of incorporation of [2-<sup>14</sup>C] mevalonate into sterols synthesized in cell-free extracts

Azole drug	IC <sub>50</sub> (nM)
Itraconazole	20.7 ± 6.5
Ketoconazole	23.2 ± 5.8
Fluconazole	79.8 ± 9.8

unknown compounds. All three azole drugs inhibited ergosterol synthesis from mevalonate in cell-free extracts. Figure 2 shows the result of a typical experiment where increasing amounts of itraconazole were added to the cell-free extracts incubated with [2-<sup>14</sup>C]mevalonate. In itraconazole containing assays there was a dose-dependent decrease in ergosterol synthesis and a corresponding increase in 14α-methyl sterols. A similar inhibitory effect was observed with fluconazole and ketoconazole. The concentration of itraconazole required to inhibit 50% incorporation (IC<sub>50</sub>) of mevalonate into ergosterol was similar to that of ketoconazole and about fourfold less than that of fluconazole (Table 2).

#### Isolation of cytochrome P450

Microsomes showed a reduced CO difference spectrum with a Soret peak at 448 nm and minor peak at approximately 420 nm which corresponds to denatured or inactive P450. A typical reduced CO difference spectrum obtained with *C. krusei* microsomes is shown in Fig. 3A. The specific content of the microsomal P450 was 26.4 ± 5.8 pmol mg<sup>-1</sup> protein. The stability of P450 was assessed by measuring a reduced CO spectrum of P450 after keeping at room temperature and it was found that *C. krusei* microsomal P450 was stable for more than 60 min (data not shown).

#### Type II difference spectra

The affinity of the azole drugs with sterol 14α-demethylase was investigated by measuring the ability of azole antifungals to induce type II difference spectra on interacting with microsomal P450. All the three drugs, itraconazole, fluconazole and ketoconazole, produced type II difference spectra. A typical type II difference spectrum obtained by adding equimolar concentration of itraconazole to microsomal P450 (0.1 μM) is shown in Fig. 3B. The spectrum exhibited a maximum absorbance at around 426 nm and a minimum at around 410 nm. The magnitude of the spectral change (the difference between maximum and minimum absorbance) was linearly dependent on the concentrations of azole drug added to the microsomal P450 (Fig. 4) and saturated at approximately equimolar concentrations of azole and P450 (0.1 μM) for ketoconazole and itraconazole. In contrast, the spectrum for fluconazole showed saturation at 0.4 μM and at equimolar concentration the spectrum was approximately 50% of the maximum response. However, the magnitude of the type II spectrum produced at the saturating concentration was the same for the all three drugs (Fig. 4). SC<sub>50</sub> values (the concentration required for inducing half saturation response) for all the three drugs are shown in Table 3. The SC<sub>50</sub> value for fluconazole is about fourfold higher than that for itraconazole and ketoconazole.

#### CO displacement studies

The relative affinity of itraconazole, fluconazole and ketoconazole with microsomal P450 was assessed by comparing their potential to delay the binding of CO to the ferrous form of iron in haem group of microsomal P450. CO displacement with the azole drugs was concentration dependent. At equimolar concentration of azole drug and P450 (0.1 μM) most of the azole drug was readily displaced by CO within 1 min (data not shown). Therefore the experiments were repeated at higher concentrations. All the drugs were able to interfere with the binding of CO, but to significantly different degrees (Fig. 5). Microsomes preincubated with fluconazole showed rapid displacement by CO. Ketoconazole was more gradually displaced with time. The same was true for itraconazole although to a much slower extent. After 60 min of incubation, the average percentage displacement efficiencies for itraconazole, ketoconazole and fluconazole were 31 ± 2, 44 ± 4 and 74 ± 7, respectively, when compared with the size of the untreated reduced CO difference spectrum.

#### Discussion

The aim of the present study was to investigate the interaction and inhibition of *C. krusei* microsomal

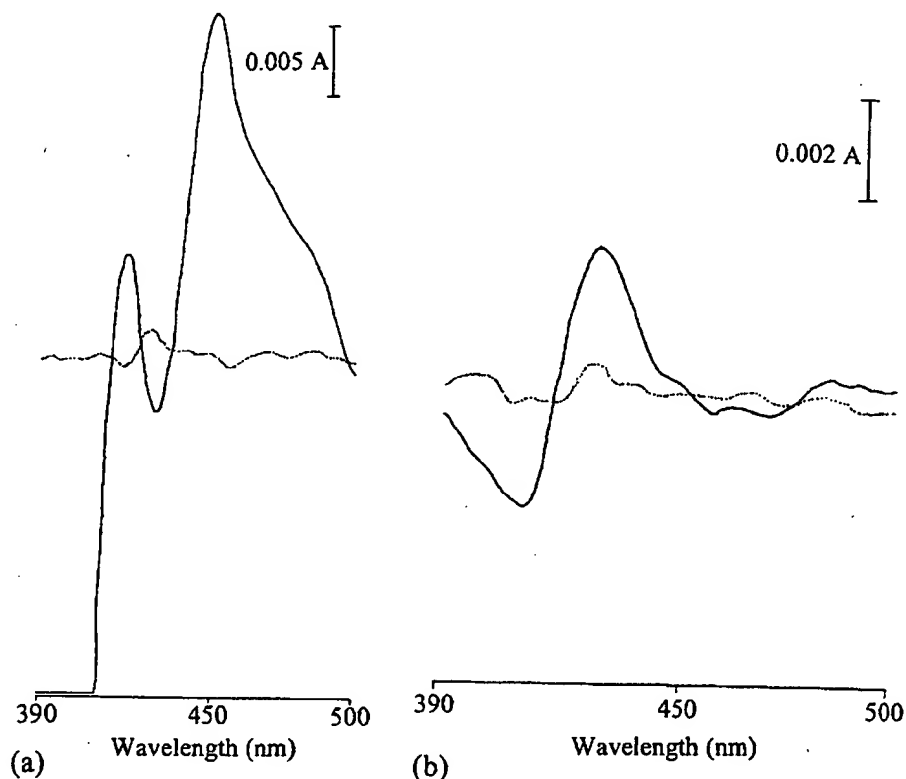


Fig. 3 (A) Typical reduced CO difference spectrum of *C. krusei* microsomal P450. (B) Type II difference binding spectrum obtained on addition of equimolar concentration of itraconazole to microsomal P450 (0.1  $\mu$ M).

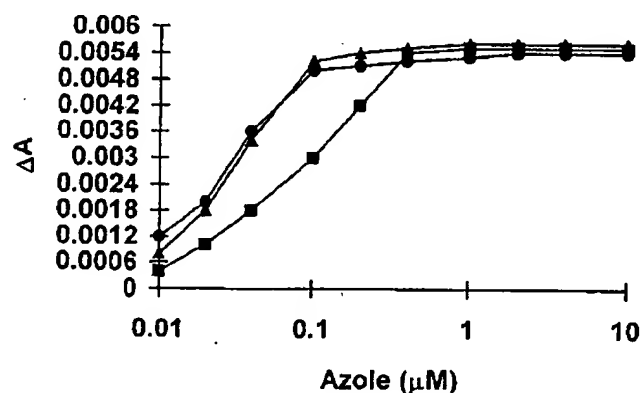


Fig. 4 Effect of concentrations of azole drugs on the magnitude of type II difference spectra of microsomal cytochrome P450. Titration curves of itraconazole (●), ketoconazole (▲) and fluconazole (■) obtained by plotting  $\Delta A$  (absorbance difference between maximum and minimum of type II difference spectra) against concentration of azole drugs.

cytochrome P450 by azole drugs. *In vitro* susceptibility studies confirmed the prior observations that ketoconazole and fluconazole are less effective than itraconazole [9]. Cell-free extracts of *C. krusei* actively synthesized ergosterol and other sterols from [2- $^{14}$ C]mevalonate. In the presence of azole there was a reciprocal relationship between ergosterol and 14 $\alpha$ -methyl sterols synthesis as observed for *C. albicans* and

Table 3 Concentrations of azole drugs which induced half saturation response ( $SC_{50}$ ) in type II difference spectrum

Azole drug	$SC_{50}$ (nM)
Itraconazole	27.6 $\pm$ 5.4
Ketoconazole	31.8 $\pm$ 3.2
Fluconazole	100.5 $\pm$ 8.5

*Saccharomyces cerevisiae* [15,17] and the  $IC_{50}$  values of itraconazole, fluconazole and ketoconazole for ergosterol biosynthesis in *C. krusei* were comparable with the values observed previously [9]. Ketoconazole was almost as active as itraconazole in inhibiting ergosterol synthesis in cell-free extracts even though it was 19 times less active in the *in vitro* cell growth inhibition. A previous report proposed that the weaker activity of ketoconazole and fluconazole on the *in vitro* growth of *C. krusei* was due to lower intracellular content of the drugs [9]. However, the microbiological resistance of *C. krusei* to fluconazole was also reflected in *in vitro* sterol biosynthesis inhibition studies. These results here support this conclusion in an isolate of *C. krusei* [9].

This study also demonstrated stable microsomal P450 isolation with a specific content of  $26.4 \pm 5.8$  pmol  $mg^{-1}$  protein, which was slightly higher than that reported for

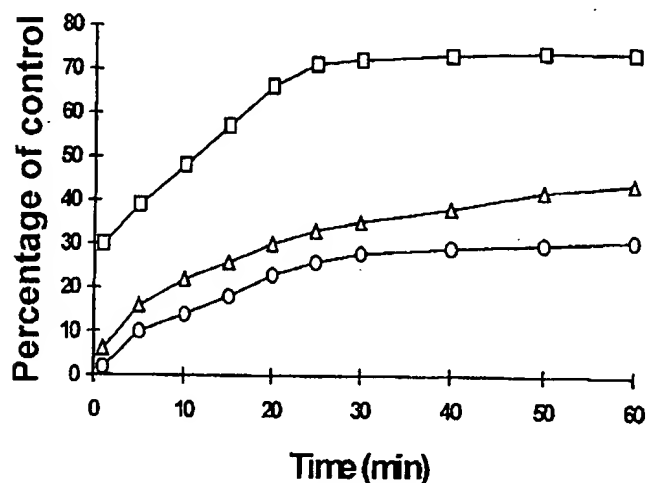


Fig. 5 Displacement of azole drugs from microsomal P450 of *C. krusei* after reduction with sodium dithionite and treatment with carbon monoxide. Displacement curves for itraconazole (○), ketoconazole (△) and fluconazole (□) were obtained by plotting  $\Delta A$  (absorbance difference between 450 nm and 490 nm of the reduced CO difference spectrum) against time after CO treatment.

*Ustilago maydis* and *A. fumigatus* but less than that reported for *C. albicans*, *S. cerevisiae* and *P. italicum* [12,13,17-19]. The age of the culture (mid log phase), gentle breakage of cells and including glycerol in the microsomal buffer were important for successful isolation of higher yields of microsomal P450. Modification of any of these parameters resulted in either low yield or a decrease in the specific content due to the breakdown of P450 to P420. Azole drugs induced type II binding spectra, characteristic of the interaction of a nitrogen atom at position three and four of the imidazole (ketoconazole) and triazole (itraconazole and fluconazole) rings, respectively, with the ferric iron of the haem of P450 as a sixth ligand [11].  $SC_{50}$  values of type II spectra demonstrated that the *C. krusei* microsomal P450 had lower affinity to fluconazole compared with ketoconazole and itraconazole as observed for *in vitro* sterol biosynthesis. The binding of ketoconazole and itraconazole to microsomal P450 saturated at equimolar concentration, indicating a close stoichiometric interaction as observed for *C. albicans* and *S. cerevisiae* but for filamentous fungi a higher concentration was required [12,17,20].

Previous studies on P450 of *S. cerevisiae* and *C. albicans* revealed that the difference in the inhibitory effect of itraconazole, ketoconazole and triadimefon on sterol 14 $\alpha$ -demethylation correlated with the ability of CO to displace these azole drugs from P450<sub>14 $\alpha$ dm</sub> [17,20]. Therefore it was suggested that displacement of azole compounds by CO from cytochrome P450 is suitable for evaluating antifungal activity of azole drugs. However, a lack of correlation between the ease of azole drugs displacement by CO and azole drugs inhibition of P450<sub>14 $\alpha$ dm</sub> has been

observed in *A. fumigatus* and *P. italicum* [12,13]. This may reflect the relative abundance of different P450s in the microsomal fraction of the fungi. In the present study itraconazole and ketoconazole were displaced from *C. krusei* microsomal P450 by CO at a slower rate than fluconazole and this observation correlated with their inhibitory effect on sterol 14 $\alpha$ -demethylation. Therefore quantitative ranking of azole antifungal compounds for inhibition of P450<sub>14 $\alpha$ dm</sub> was possible using the drug interaction studies with the microsomal P450 of *C. krusei* and may have further general applications in structure-activity relationship studies for rational drug design. Based on experience in other fungal species [15,19] similar levels of enzyme and responses to azole compounds are anticipated in other isolates of *C. krusei*. However, each of the *in vitro* findings has only a narrow application and the sum of all of these, plus the pharmacokinetic parameters determine the overall efficacy of the drug.

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